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In silico predicted conserved B-cell epitopes in the merozoite surface antigen-2 family of *B. bovis* are neutralization sensitive

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ABSTRACT

The merozoite surface antigens MSA-2 of *Babesia bovis* constitute a family of polymorphic GPI-anchored glycoproteins located at the parasite cell surface, that contain neutralization-sensitive B-cell epitopes. These are therefore putative vaccine candidates for bovine babesiosis. It was previously shown that (i) the MSA-2 antigens of the biologically cloned Mo7 strain are encoded by four tandemly organized genes: *msa-2a*$_1$, *a*$_2$, *b* and *c*, and (ii) at least one allele of each of these genes is present in the Argentine R1A strain with a moderate degree of polymorphism. The present work was aimed at defining neutralization-sensitive B-cell epitopes in the MSA-2 family, that are conserved among different *B. bovis* geographical isolates. To this end, *msa-2a*, *b* and *c* alleles from different isolates from Argentina, USA and Mexico were amplified by PCR, cloned and sequenced. Bioinformatic analysis by ClustalW alignments and B-cell epitope prediction algorithms performed on these sequences allowed the identification of several regions containing putative conserved B-cell epitopes. Four peptides representing these regions: (KDYKTMVKFCN from *msa-2a*$_1$; YYKKHIS, from *msa-2b*; and THDALKAVKQLIKT and ELLKLLIEA from *msa-2c*) were chemically synthesized, conjugated to keyhole limpet hemocyanin and used to inoculate mice to obtain immune sera. Anti-peptide antibodies recognized *B. bovis* merozoite extracts in all cases in ELISA tests. In addition, these sera reacted with the surface of merozoites of an Argentine and a Mexican *B. bovis* strains in immunofluorescence assays, and sera against two of the selected peptides inhibited invasion of erythrocytes by *in vitro* cultured merozoites. Taken together, the results show that the peptide sequences selected by bioinformatic analysis represent expressed and geographically conserved *B. bovis* B-cell epitopes that might be strong candidates for development of subunit vaccines.

1. Introduction

Infection of bovines with the tick-transmitted hemoparasite *Babesia bovis* causes important economic losses and limits cattle production in vast tropical and subtropical areas of the world. An effective control measure is vaccination of unprotected cattle with live attenuated

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strains, which allows the prevention of outbreaks in areas of enzootic instability and protects animals raised in tick-free zones when imported into endemic areas (Shkap et al., 2007). These vaccines are usually effective but have a number of disadvantages, such as the risk of co-transmission of blood-borne pathogens, the need of a cold chain, a relatively short storage life when it is not frozen, and the danger of parasite reversion of virulence. In addition, they are only safe in bovines younger than 1 year old. Some reports have pointed out sporadic failures of live vaccines (Bock et al., 1992, 1995), which could be due to the occurrence of variant parasite strains against which the vaccines do not generate protection.

Development of subunit vaccines could prove useful for the control of bovine babesiosis. Experimental vaccines based on single recombinant antigens have been successfully developed for some apicomplexan hemoparasites of veterinary importance. Such is the case for *Theileria parva*, where vaccination of cattle with a recombinant form of the sporozoite surface antigen p67 significantly reduced the development of East Coast Fever after a natural tick challenge in Kenya (Musoke et al., 2005). Likewise, immunization of gerbils with a recombinant form of Bd37, a glycosyl-phosphatidylinositol (GPI)-anchored protein of *B. divergens*, induced protection against challenge with different virulent strains of this parasite (Hadj-Kaddour et al., 2007). However, attempts to elicit protective immune responses against *B. bovis* using single recombinant proteins derived from known strong immunogenic proteins such as MSA-1 and RAP-1 proved to be so far unsuccessful (Hines et al., 1995; Norimine et al., 2003). A recombinant vaccine based on the *B. bovis* antigens 11C5 and 12D3 was recently tried with encouraging results (Hope et al., 2005), although full protection was not achieved. Thus, these results suggest that a protective *B. bovis* subunit vaccine may require the inclusion of several antigens or alternatively, a combination of effective B- and T-cell epitopes derived from multiple antigens.

Promising candidates for *B. bovis* subunit vaccines include members of the merozoite surface antigen (MSA)-2 family. This family was originally identified in transcripts from the virulent Kv Australia strain and termed *Babesia* recombining genes (BabR) (Cowman et al., 1984). The gene family was afterwards identified and their products characterized in North American *B. bovis* strains. Interestingly, members of this family contain a proline-rich hypervariable region and present a considerable degree of sequence variation among strains, although they do not seem to change rapidly during the course of infection, and thus the family was renamed as variable merozoite surface antigen (VMSA), including the MSA-1 and MSA-2 genes (Reduker et al., 1989; Hines et al., 1989, 1992; Berens et al., 2005; LeRoith et al., 2006). They are a group of immunodominant, GPI-anchored glycoproteins, homogeneously distributed over the surface membrane of both merozoites and sporozoites (Reduker et al., 1989; Hines et al., 1989; Mosqueda et al., 2002). Importantly, VMSA proteins were shown to contain neutralization-sensitive B-cell epitopes and are thought to participate in erythrocyte invasion (Hines et al., 1992; Suarez et al., 2000; Florin-Christensen et al., 2002; Mosqueda et al., 2002; Wilkowsky et al., 2003). Definitive characterization of the genomic *msa-2* locus in the Mexican Mo7 strain (Florin-Christensen et al., 2002) showed a tandem organization of four related genes, which were named: *msa-2a*$_1$, *msa-2a*$_2$, *msa-2b* and *msa-2c*. A similar structure was later found in the virulent Texas T2Bo strain (Berens et al., 2005; Brayton et al., 2007). At least one allele of each *msa-2* gene was identified in the Argentine R1A strain, and comparison of R1A and Mo7 sequences showed a moderate to high degree of conservation (Florin-Christensen et al., 2002). However, characterization of the *msa-2* locus in 12 Australian strains demonstrated a completely different structure and, with the exemption of *msa-2c,* a high degree of polymorphism among strains (Berens et al., 2005).

A variable B-cell epitope recognized by the monoclonal antibody 23/70.174 was identified and mapped in a repetitive region of MSA-2 in the Texas and Mexico strains of *B. bovis* MSA-2a (Goff et al., 1988; Palmer et al., 1991; Jasmer et al., 1992). However, the moderate degree of sequence conservation later found between R1A and Mo7 MSA-2 predicted proteins allowed us to hypothesize that this family might contain other B-cell epitopes that, in contrast to the epitope recognized by mAb 23/70.174, are conserved among American geographic isolates. This work has been aimed at identifying and characterizing such B-cell epitopes, with the long-term goal of developing a subunit vaccine that would be effective against otherwise antigenically different strains of *B. bovis*.

2. Materials and methods

2.1. Strains and isolates

The following *B. bovis* strains and isolates were used in this study: Mo7, RAD, Pullman, Veracruz and Tabasco, from Mexico; R1A, S2P, M1A, M2P, and M3P from Argentina and T2Bo from USA. Mo7, a biologically cloned Mexican strain and the isolates T2Bo (Texas, USA), R1A (vaccine strain initially isolated from a clinical case in Santa Fe, Argentina in 1990 and attenuated across passages in splenectomized bovines, Anziani et al., 1993), and S2P (pathogenic isolate from Salta, Argentina) were propagated in cultured bovine erythrocytes. The M2P and M3P isolates derive from clinical cases in the province of Corrientes, Argentina, and were amplified in splenectomized calves. M1A was isolated from a clinical case in Salta and attenuated after several passages in splenectomized calves in Mercedes, Argentina. It is used for vaccination in the NE of this country. The Mexican strains RAD (vaccine strain), Veracruz and Tabasco derive from clinical cases and were propagated *in vitro* or amplified in splenectomized bovines. The Pullman isolate was obtained from infected ticks in Mexico, 1975, and was amplified in splenectomized bovines as well.

2.2. PCR, cloning and sequencing

Genomic DNA was purified by standard phenol/chloroform extraction. In the case of *in vitro* cultured parasites, *B. bovis* merozoite-enriched suspensions were first obtained by differential centrifugation (4000 × *g*, 20 min, and 10,000 × *g*,

20 min) after 1 h incubation of cultures at 4 °C. In the case of field isolates, whole blood was frozen at −20 °C, thawed and centrifuged (10,000 × *g*, 30 min). The pellet was washed twice with PBS, and then used for DNA extraction. Amplification of *msa-2a1*, *2b* and *2c* from these DNA samples was carried out by PCR. The complete open reading frames (ORF) of *msa-2a1* and b were amplified together using the oligonucleotides B44F (5′-ATGATCGGGAAAATCTTC-3′) and B42/44R (5′-AAAATGCAGAGAGAACG-3′). For amplification of the *msa-2c* complete ORF, the oligonucleotides: MSA-2cF (5′-ATGGTGTCTTTTAACATAATAAC-3′) and B42/44R were employed. PCR was carried out for 35 cycles (1 min at 95 °C, 1 min at 55 °C, 2 min at 72 °C), followed by 4 min at 72 °C. Amplification was confirmed on ethidium bromide-stained agarose gels. Amplicons (∼930 bp for *msa-2a1*, ∼860 bp for *msa-2b* and ∼790 bp for *msa-2c*), were cloned into pCR TOPO 2.1 (Invitrogen, Carlsbad, CA). Three to five clones were randomly selected and plasmids were purified for sequencing using QIAprep Spin Miniprep Kit (Qiagen, Valencia, LA). Sequencing was carried out by Macrogen Inc. (Seoul, South Korea), using M13 forward and reverse primers. New sequences were deposited in the Genbank with the following Accession Numbers: M2P *msa-2c*: FJ411372; M3P *msa-2c*: FJ422794; M1A *msa-2c*: FJ422795, S2P *msa-2c*: FJ422796, Pullman *msa-2c*: FJ422797, Tabasco *msa-2c*: FJ422798, RAD *msa-2c*: FJ422799, Veracruz *msa-2c*: FJ422800, RAD 1 *msa-2b*: FJ422801; RAD 2 *msa-2b*: FJ422802; Tabasco *msa-2b*: FJ422803; M1A *msa-2b*: FJ422804; M2P *msa-2b*: FJ422805; M2P *msa-2a1*: FJ422806; S2P *msa-2a1*: FJ422807; M3P *msa-2a1*: FJ422808, Veracruz *msa-2a1*: FJ422809. In addition, the following sequences already deposited in the Genbank were used: R1A *msa-2c*: AAL15428, Mo7 *msa-2c*, *b* and *a1*: AY052538, T2Bo *msa-2c*: DQ173974, R1A *msa-2b*: AAL15427, T2Bo *msa-2b*: DQ173961, R1A *msa-2a1*: AAL15425, T2Bo *msa-2a1*: DQ173959.

2.3. Bioinformatic analysis of sequences

Sequences were analyzed and compared using the BioEdit 7.0.5.3 version software (Hall, 1999) and the Baylor College of Medicine website (http://searchlauncher.bcm.tmc.edu). Multiple alignments were carried using the software of the European Bioinformatics Institute website (http://www.ebi.ac.uk/Tools/clustalw2/); and pairwise sequence alignments with a BLOSUM matrix from http://searchlauncher.bcm.tmc.edu/seq-search/alignment.html, with a gap penalty of 12, and a gap extension penalty of 4. Sequence comparison graphs were constructed using Boxshade 3,2 from Embnet, Swiss Institute of Bioinformatics (http://www.ch.embnet.org/software/BOX_form.html). Signal peptide and GPI-anchor cleavage sites were predicted using the algorithms of the websites: http://www.cbs.dtu.dk/services/SignalP and http://mendel.imp.ac.at/home/Birgit.Eisenhaber, Fungi option, respectively. Secondary structures were predicted using CLC Combined Workbench 3 software, available at http://www.clcbio.-com/index.php?id=92); and B-cell epitopes, by the method of Kolaskar and Tongaonkar (1990) (http://bio.dfci.harvard.edu/Tools/antigenic.pl).

2.4. Peptide synthesis

The degrees of antigenicity, hydrophilicity, solubility, stability and synthesis difficulties of all peptides containing predicted B-cell epitopes were calculated using the website: www.sigma-genosys.com/calc/pepCalc.asp. Peptides 1 (ELLKLLIEA), 2 (YYKKHIS), 3 (KDYKTMVKFCN) and 4 (THDALKAVKQLIKT) were chemically synthesized with 80% purity by Genbiotech (Buenos Aires, Argentina). Peptides were acetylated in the amino terminal ends, and contained three glycines and one cysteine added to their carboxyl termini for linking to the keyhole limpet hemocyanin (KLH) carrier. In the case of Peptide 3, the final D residue was omitted, to avoid formation of a bridge with the glycines. Linking of peptides to KLH was carried out by the same company. All peptides were predicted to be water-soluble. However, peptides 3 and 4 were insoluble. Dimethylsulfoxide/water 1:7 (v/v) was used as solvent in these cases. The concentration of peptide stock solutions was 10 mg/ml.

2.5. In vitro culture of B. bovis merozoites

B. bovis R1A merozoites were *in vitro* grown basically as described by Levy and Ristic (1980), in a medium containing 10% erythrocytes and 40% normal bovine serum in M199 medium (Gibco, Sigma Chemical Co., St. Louis, MO), supplemented with 17 mM HEPES (Sigma Chemical Co.), 100 μg/ml streptomycin, 100 UI/ml penicillin, pH 7.2. Cultures were grown in 24-well plates (in a depth of 5 mm), at 37 °C in a humidified 5% CO_2 atmosphere, with daily renovation of the overlaying medium. Percentages of infected erythrocytes (PPE) were estimated by microscopic examination of 2500 erythrocytes in Giemsa-stained smears. Subcultures were started when the PPE was 5% or more.

2.6. Production of anti-peptide sera

Antisera to the four KLH-conjugated peptides were raised in mice ($n = 10$ for each peptide). Mice received four intraperitoneal injections of 200 μl of immunogen containing 100 μg of KLH-conjugated peptide emulsified in complete Freund's Adjuvant (Sigma Chemical Co.), at day 0 or 50 μg in incomplete Freund's Adjuvant, at days 14, 35 and 56. Tail blood was collected at day 70 and the antisera used in ELISA, neutralization and immunofluorescence studies. Sera of mice ($n = 5$) inoculated with PBS under the same conditions were used as negative control.

2.7. Production of anti-merozoite sera

In vitro grown *B. bovis* merozoites (R1A strain) were isolated from erythrocytes using a Percoll gradient, as described previously (Rodriguez et al., 1986), suspended in PBS, lysed by sonication and centrifuged (12,000 × *g*, 5 min, 4 °C). Three BALB/c mice were subcutaneously injected with 30 μg each of the supernatant, containing solubilized merozoite antigens, emulsified in PBS-Montanide-50 (Seppic-Montanide, Paris, France), on days 0, 15 and 30. Mice were bled on day 40 and sera were stored at −20 °C until use.

2.8. Production of MSA-2b and 2c recombinant proteins and sera against them

Recombinant forms of *B. bovis* R1A MSA-2b and MSA-2c were produced in *E. coli* using the pBAD/thioTOPO (Invitrogen) prokaryotic expression system, and purified by affinity chromatography in Ni-agarose as previously described (Wilkowsky et al., 2003). Antisera were raised in mice ($n = 3$ for each protein) intraperitoneally injected with 25 μg of rMSA-2b or rMSA-2c, emulsified in Freund's Adjuvant as described for anti-peptide sera. After boosters at days 14 and 28, blood was collected from the tails on day 42; and sera were stored at −20 °C until use.

2.9. Merozoite indirect ELISA

An assay developed by Echaide et al. (2004) was developed. Briefly, a purified suspension of *in vitro* grown merozoites was diluted in 0.6 M sodium carbonate/sodium bicarbonate buffer, pH 9.6, and coated on Nunc-PoliSorp multiwell plates (Thermo Fisher Scientific, Franklin, MA). Plates were incubated overnight at room temperature, and blocked with PBS/0.05% Tween-20 (PBST) containing 4% milk. Sera (1/50 dilution in PBST/0.02 M EDTA/0.015 M EGTA, pH 6.3) were added to the plates, and incubated for 30 min at 37 °C with shaking, followed by four PBST washes. Determination of total IgG antibodies was carried out by incubation with a previously titered dilution of peroxidase-labeled goat anti-mouse IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA) followed by plate washes, and 1 mM 2-2′-sulfonic azino-bis-3-ethylbenzothiazoline-6-acid (ABTS, Sigma Chemical Co.)/0.015% H_2O_2 in 0.05 M sodium citrate buffer, pH 4.5. Results were recorded when the absorbance reading at 405 nm of the plate reached a value between 0.8 and 1.2. The mean value of triplicate determinations was calculated. Results are expressed as the percentage of this mean with respect to the mean absorbance value obtained with three anti-merozoite sera, raised in mice (percentage of positivity, % *P*). The cut-off value (60%) was calculated as the average of 35 negative sera plus 3 standard deviations. The anti-merozoite serum sample that yielded the highest absorbance measurement and the serum sample of the group inoculated with PBS, which yielded the lowest absorbance were used as positive and negative controls, respectively, in the *in vitro* neutralization experiment. The latter was also used as negative control in the fixed immunofluorescence antibody test.

2.10. Fixed immunofluorescence antibody test (IFAT)

IFAT was performed as described previously (de Ríos et al., 1988), to assess the reactivity of anti-peptide sera towards fixed *B. bovis* R1A and RAD merozoites. Monoclonal antibody PB/5 (Sahagun Ruiz et al., 2000), that recognizes a 152 kDa *B. bovis* intracellular protein of apical localization, was included as a positive control, and MAb Tryp1E1, that is reactive with a variable surface glycoprotein of *Trypanosoma brucei*, as well as serum of a mouse inoculated with PBS were used as negative controls. A previously titrated dilution of Alexa Fluor-488-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA) was used as secondary antibody. Probe-on slides (Fisher Scientific, Pittsburgh, PA) were mounted with 1,4-diazobicyclo(2,2,2)-octane (DABCO, Sigma Chemical Co.), and observed under fluorescence microscopy (1000× magnification).

2.11. In vitro neutralization assay

Experiments of erythrocyte invasion in the presence of different sera were essentially carried out as previously described (Wilkowsky et al., 2003), Briefly, aliquots of 5×10^5 live purified *B. bovis* merozoites (R1A strain) were incubated in 96-well plates, in triplicates, with different heat-inactivated (56 °C, 30 min) mice sera: anti-rMSA-2c, anti-rMSA-2b and anti-merozoite sera (positive controls); immune sera against peptides ELLKLLIEA (1), YYKKHIS (2), KDYKTMVKFCN (3) and THDALKAVKQLIKT (4); and serum of a mouse inoculated with PBS under the same conditions (negative control). All sera were diluted 1/5 in culture medium. After an incubation period of 30 min at 4 °C, an equal volume of 5% (v/v) bovine erythrocytes in culture medium was added to each well and the plates were incubated at 37 °C in a 5% CO_2 atmosphere. At 24, 48 and 72 h, the supernatant (80% of the total volume) was replaced with fresh culture medium. PPE were determined at 96 h. The statistical significance of the differences between the PPE mean obtained with each tested serum and the PPE mean obtained with control serum was determined by the Student's *t* test.

3. Results and discussion

We first analyzed and compared the predicted amino acid sequences of MSA-2a$_1$ ($n = 7$), MSA-2b ($n = 8$) and MSA-2c ($n = 11$) proteins from different *B. bovis* American isolates. The results are shown in Fig. 1. High conservation was found in the amino and carboxyl termini, which correspond to the signal peptide (amino acids 1–22 for MSA-2a1 and b and 1–21 for MSA-2c) and GPI-anchor signal (final stretch of 21 amino acids), respectively. Both peptides are likely absent in the mature proteins, but they were included for convenience in the alignments.

Fig. 1A presents an alignment of the MSA-2a1 predicted proteins analyzed. They show a moderate to high degree of conservation among isolates, ranging from 68 to 96% of identity with respect to the R1A sequence. Substitutions are distributed quite evenly along the sequences and some small gaps are found close to the carboxyl terminus. Fig. 1B shows the alignment of MSA-2b proteins. Interestingly, two distinct *msa-2b* sequences were amplified from the RAD strain (named RAD1 and RAD2 in the alignment), sharing 85.7% identity towards each other in their predicted amino acid sequences. Since RAD is a clonal strain, these results suggest that the RAD genome contains at least two *msa-2b* paralogous genes. The degree of identity of MSA-2b peptide sequences range from 70 to 84%. Most noticeably, three of the sequences (M1A, M2P and Tabasco) show two consecutive gaps of 15 and 12 residues each, spanned by a 7-residue island (SAAPNTS) in a proline-rich region close to the carboxyl terminus. These results coincide with the observations of Berens et al.

(2005) who described a hypervariable region in this area for MSA-2 proteins from American and Australian isolates. In addition, LeRoith et al. (2006) also found a proline-rich hypervariable region in another member of the VMSA family, MSA-1, which is likely exposed to the surface and contains neutralization-sensitive epitopes. It has been suggested that proline residues could participate in the structural comformation of these antigens (LeRoith et al., 2006).

MSA-2c proteins are the shortest and most conserved members of the MSA-2 family (Fig. 1C), with percentages of identity between 86 and 94% with respect to R1A. These

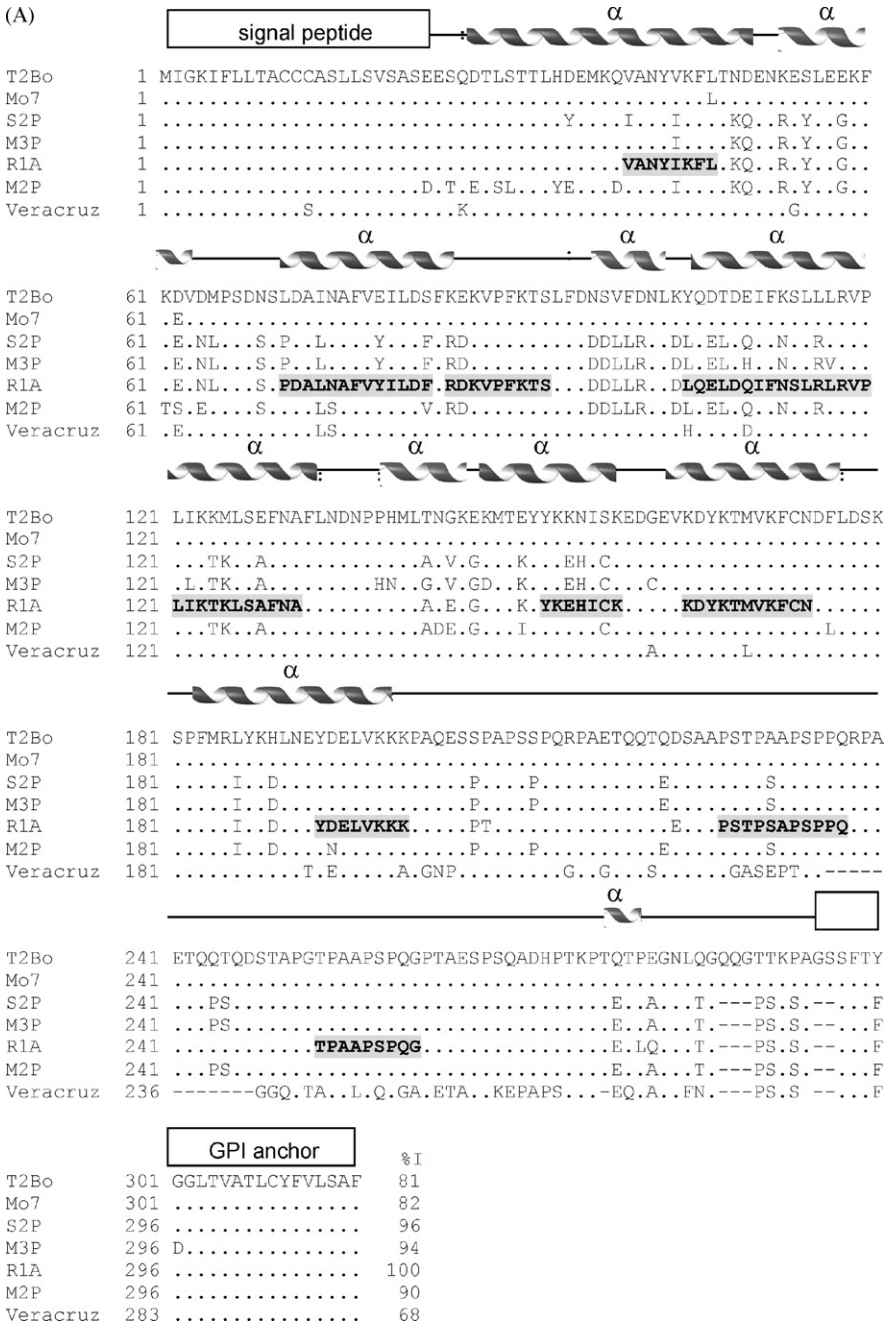

Fig. 1. Comparison of *msa-2* predicted amino acid sequences from *Babesia bovis* geographical isolates. MSA-2a$_1$ (A), b (B) and c (C) alleles from different American isolates were PCR-amplified, cloned, and sequenced. Predicted amino acid sequences were aligned using ClustalW and Boxshade. Dots in the alignments correspond to conserved amino acids, and short lines to gaps. B-cell epitopes, predicted for the sequences obtained for R1A, are shown with the corresponding residue letters and shaded in gray. The predicted secondary structure of the mature R1A proteins are shown at the top of the sequences. α: α-helix; β: β-sheet. Lines correspond to stretches of amino acids with no predicted structure. Identity percentages (%I) with respect to R1A peptide sequences are shown at the end of each sequence.

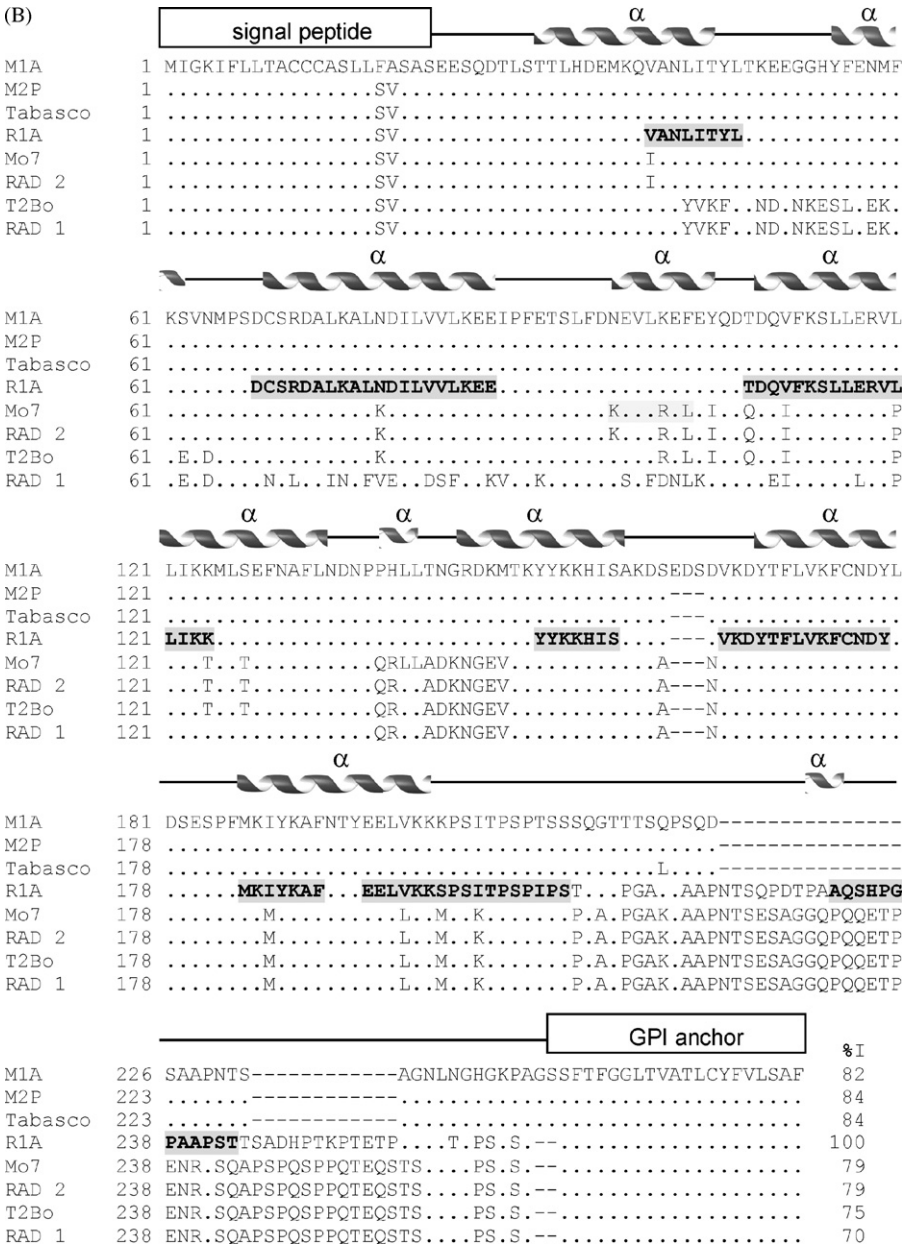

Fig. 1. (*Continued*).

results agree well with previous reports (Florin-Christensen et al., 2002) and studies performed on Australian isolates (Berens et al., 2005).

Fig. 1A, B and C also show the predicted α-helices for the R1A MSA-2 proteins. A tandem arrangement of 9–10 α-helices spanned by short stretches of non-structured sequence can be observed. Only small changes in this predicted structure were found in the different MSA-2 proteins from other strains analyzed (not shown), and could be associated with amino acid substitutions in the same or neighboring regions. Such changes consisted in, for example, the disappearance or appearance of a short α-helix or the division of a longer α-helix into two smaller ones, or conversely, the joining of two separate α-helices into a single one. The most remarkable observation of this analysis is that the structure of a α-helix bundle is conserved in MSA-2a1, b and c proteins of all isolates despite extensive amino acid variation, suggesting that this predicted type of folding might be essential for their functionality.

B-cell epitope prediction analysis was carried out for the R1A MSA-2a1, 2b and 2c putative proteins, using the Kolaskar and Tongaonkar method (1990). The algorithm employed in this method is based on hydrophilicity, aminoacidic environment, and comparison with already described epitopes in other species. We identified nine

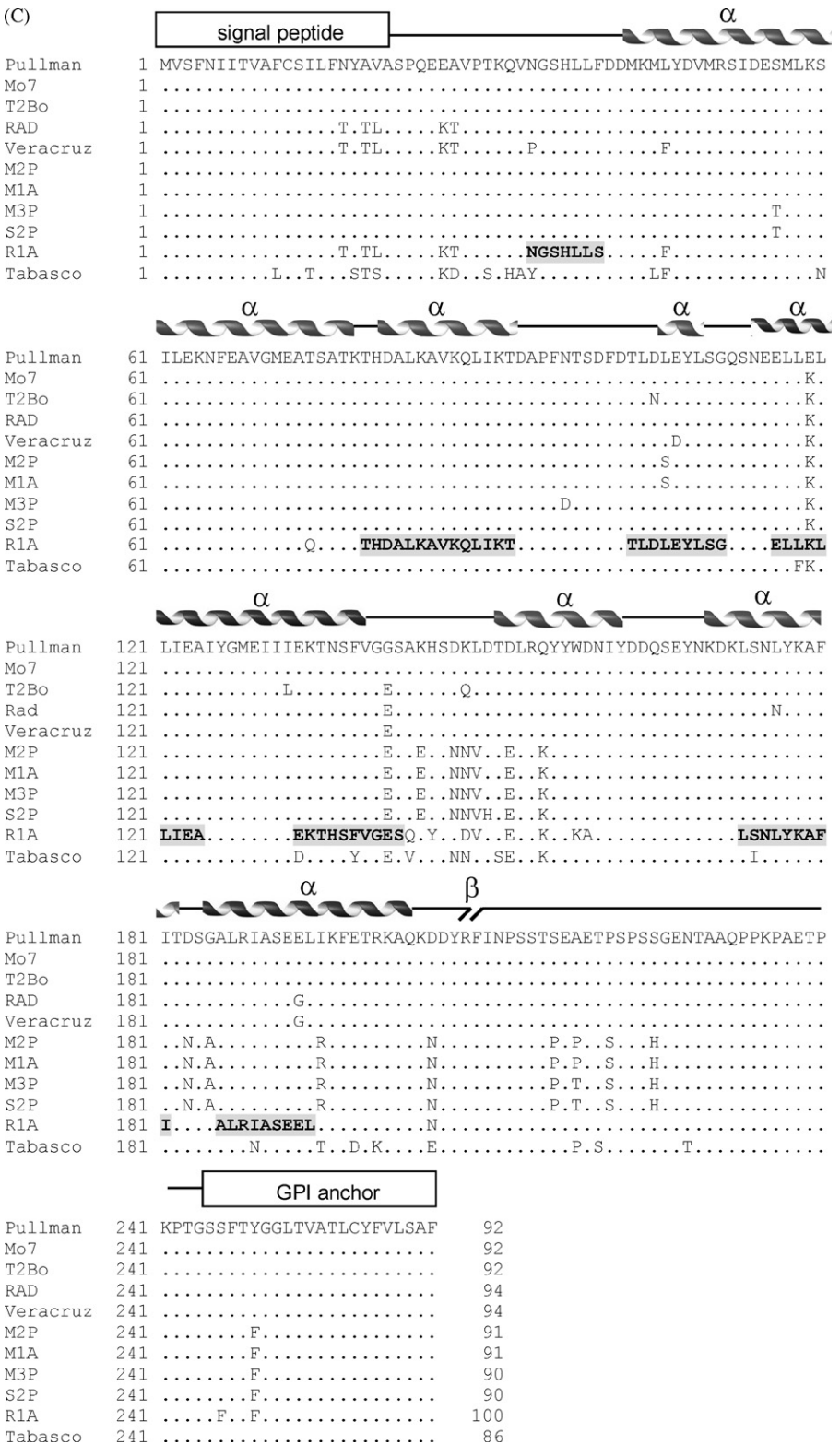

Fig. 1. (*Continued*).

Table 1
Predicted B-cell epitopes in MSA-2 peptide sequences of the *Babesia bovis* R1A strain.

Protein of origin	Predicted B-cell epitopes	Conserved amino acids/Total
MSA-2a1	VANYIKFL	6/8
	PDALNAFVYILDF	8/13
	RDKVPFKTS	7/9
	LQELDQIFNSLRLRVPLIKTKLSAFNA	16/27
	YKEHICK	4/7
	KDYKTMVKFCN (Peptide 3)	10/11
	YDELVKKK	6/8
	PSTPSAPSPPQ	3/11
	TPAAPSPQG	4/9
MSA-2b	VANLITYL	3/8
	DCSRDALKALNDILVVLKEE	9/20
	TDQVFKSLLERVLLIKK	11/17
	YYKKHIS (Peptide 2)	7/7
	VKDYTFLVKFCNDY	14/14
	MKIYKAF	6/7
	EELVKKSPSITPSPIPS	12/17
	AQSHPGPAAPST	1/12
MSA-2c	NGSHLLS	5/7
	THDALKAVKQLIKT (Peptide 4)	14/14
	TLDLEYLSG	6/9
	ELLKLLIEA (Peptide 1)	7/9
	EKTHSFVGES	6/10
	LSNLYKAFI	7/9
	ALRIASEEL	7/9

putative B-cell epitopes in MSA-a1; 8, in MSA-2b and 7, in MSA-2c (Table 1 and Fig. 1A, B and C). In most cases, B-cell epitopes coincide with α-helices, but not every α-helix contains predicted epitopes.

Four regions among these proteins (1 [*msa-2c*]: ELLKLLIEA; 2 [*msa-2b*]: YYKKHIS; 3 [*msa-2a1*]: KDYKTMVKFCN and 4 [*msa-2c*]: THDALKAVKQLIKT) were chosen for developing synthetic peptides for further studies, because they met one or more of the following criteria: high conservation among isolates (Table 1), high predicted antigenicity, and high predicted accessibility (not shown). Peptides were chemically synthesized, bound to KLH, and inoculated in mice ($n = 10$ for each). Reactivity of each mouse immune serum against *B. bovis* R1A merozoite extracts was analyzed by indirect ELISA (Fig. 2). Some serum samples in every group reacted with R1A merozoites, showing that the B-cell epitopes predicted *in silico*, are indeed expressed in the parasites, and that the antibodies raised against the synthetic peptides are able to recognize parasite epitopes.

Recognition of the parasite surface by the four anti-peptide sera was tested by IFAT using smears of erythrocytes infected with merozoites either of the Argentine R1A or the Mexican RAD strains. Four serum samples that recognized merozoite extracts on ELISA plates were selected for this experiment (marked with arrows in Fig. 2). The four anti-peptide sera reacted with the parasite surface in both distinct strains, resulting in homogenous fluorescence patterns of reactivity in all cases (Fig. 3 for anti-peptide 1 and 4, and results not shown for anti-peptide 2 and 3).

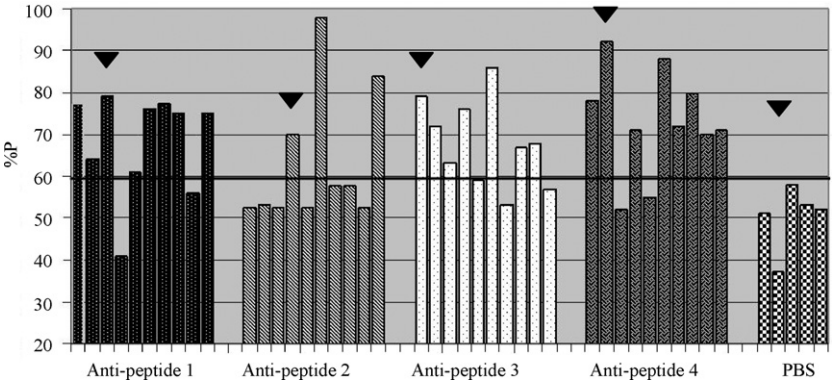

Fig. 2. *Reaction of murine anti-peptide sera with Babesia bovis merozoite proteins in an indirect ELISA.* Results are expressed as percentages of positivity (% *P*) obtained for each individual serum sample, considering as 100% the absorbance value obtained with a reference positive serum, as described in Materials and Methods. A cut-off of 60% was established calculating the average % *P* value of 35 negative mouse sera, plus 3 standard deviations. Peptide 1: ELLKLLIEA (from MSA-2c), Peptide 2: YYKKHIS (from MSA-2b); Peptide 3: KDYKTMVKFCN (from MSA-2a1); Peptide 4: THDALKAVKQLIKT (from MSA-2c). The triangles point at the sera that were used in the IFAT and neutralization experiments.

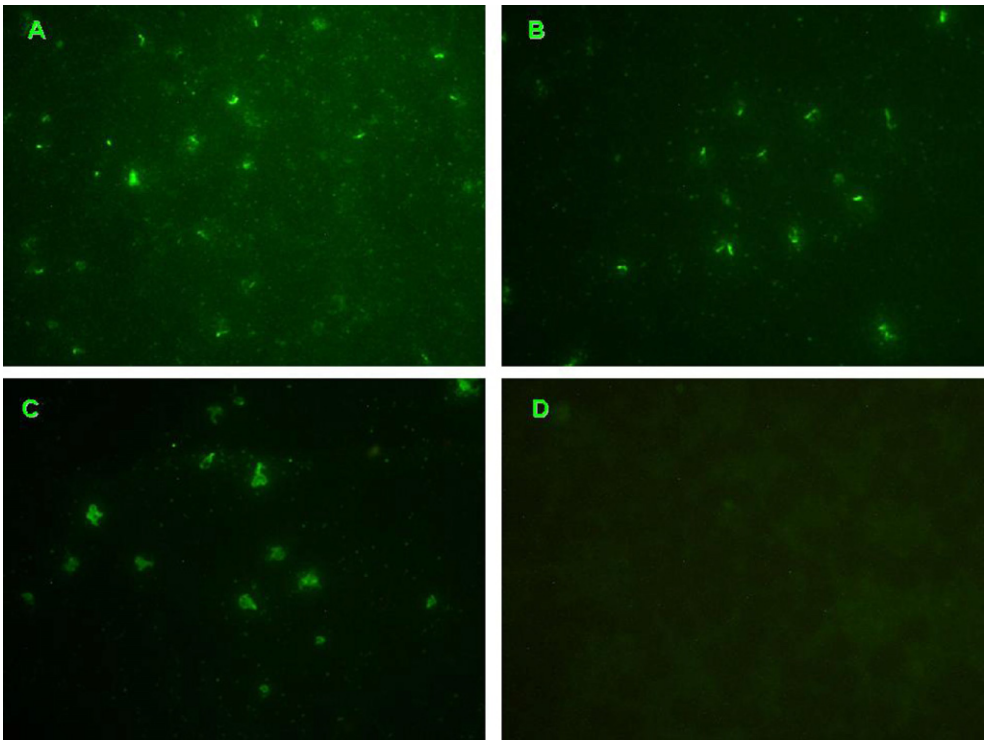

Fig. 3. *Recognition of Babesia bovis merozoite surface by murine anti-peptide antibodies.* Fixed immunofluorescence antibody test (1000× magnification) shows the reactivity with the surface of *B. bovis* strain RAD merozoites of (A) murine anti-peptide 1 antibodies (reactive with MSA-2b), (B) murine anti-peptide 2 antibodies (reactive with MSA-2a1). (C), and anti-rMSA-2c antibodies, and (D) negative control murine serum. Sera were obtained at 70 days post inoculation.

Taken together, these results (i) confirm that the predicted B-cell epitopes are indeed expressed in the parasites, (ii) demonstrate that the anti-peptide antibodies are able to recognize the native parasite proteins, (iii) indicate that these predicted B-cell epitopes are homogeneously distributed on the merozoite surface membrane, (iv) show that they are conserved between two geographically distant American isolates, since anti-peptide antibodies reacted with both R1A and RAD merozoites and (v) the selected peptides contain actual B-cell epitopes, since they were all able to elicit antibody responses in mice.

Finally, we evaluated the capacity of the four anti-peptide sera used above to inhibit merozoite invasion of erythrocytes. Briefly, R1A merozoites were partially purified, incubated with the different anti-peptide or control sera, and re-incubated with a new batch of erythrocytes, under *in vitro* culture conditions that allow growth. As positive controls, murine sera raised against a whole R1A merozoite extract and sera against rMSA-2b and rMSA-2c were used. The serum of a mouse inoculated with PBS was used as negative control. Percentages of parasitized erythrocytes were obtained for each condition after 96 h of culture (Table 2). It can be observed that when merozoites had been incubated with sera against Peptides 2 (YYKKHIS, derived from MSA-2b) and 3 (KDYKTMVKFCN, derived from MSA-2a1), the PPE values obtained were significantly lower. The inhibition of erythrocyte invasion for anti-peptide 2 and 3 sera was of 73.3 and 66.6%, respectively. Thus, the data indicate that Peptides YYKKHIS and KDYKTMVKFCN correspond to neutralization-sensitive B-cell epitopes.

Table 2 shows that murine antibodies against full size rMSA-2b, used as positive control, hampered erythrocyte invasion. This result coincides with previous work by Mosqueda et al. (2002). Full size MSA-2a1 has also been shown to contain neutralization-sensitive B-cell epitopes by Hines et al. (1992) and Mosqueda et al. (2002). However, our work is the first to map two neutralization-sensitive B-cell epitopes in MSA-2a1 and MSA-2b that, remarkably, are conserved among Mexican and Argentine strains.

It is noteworthy that the sequence of Peptide 2 (YYKKHIS) coincides with the most hydrophilic peak predicted for the MSA-2b antigen (Florin-Christensen et al., 2002). Interestingly, the motifs YYK and VKFCN, present in Peptides 2 and 3, respectively, show absolute conservation in MSA-2a/b peptide sequences from Australian breakthrough isolates (Berens et al., 2005).

Conservation of surface exposed B-cell epitopes, which are permanently exposed to the selection pressure of the host immunological system, could be indicative of a physiological constraint to variation. In particular, those protein regions containing conserved neutralization-sensitive B-cell epitopes are attractive targets for vaccine development.

In addition, the sequence ANYIKFLT is also highly conserved among MSA-2a1 variants of American isolates

Table 2
Effect of anti-peptide sera on the invasion of erythrocytes by Babesia bovis merozoites. Partially purified merozoites of the R1A strain were exposed to the following murine sera: (i) sera from mice inoculated with PBS as a negative control (PBS control); (ii) anti-peptide sera; (iii) sera against recombinant forms of MSA-2b and 2c (anti-rMSA-2b and anti-rMSA-2c) and (iv) sera of mice inoculated with a *B. bovis* (R1A) merozoite protein extract (anti-merozoite). After this exposure, merozoites were incubated with normal bovine erythrocytes under normal culture conditions. After 96 h, percentages of parasitized erythrocytes (PPE) were determined by microscopic observation of Giemsa-stained smears. Results shown are the averages obtained in triplicate parallel wells. Asterisks in brackets show significantly decreased PPE values with respect to PBS control samples. (*) $p < 0.005$, (**) $p < 0.01$.

Serum	PPE
PBS control	4.5
Anti-peptide 1	4.7
Anti-peptide 2	1.2 (*)
Anti-peptide 3	1.5 (*)
Anti-peptide 4	6.4
Anti-merozoite	1.5 (*)
Anti-rMSA-2c	0.8 (*)
Anti-rMSA-2b	2.1 (**)

(Fig. 1A) and MSA-2a/b variants of Australian isolates (Berens et al., 2005), and contributes 7 of the 8 amino acids present in one of the B-cell epitopes predicted in this study (VANYIKFL, Table 1). Further research will be devoted to test if this peptide also corresponds to a neutralization-sensitive B-cell epitope.

Sera against peptides 1 and 4, both derived from MSA-2c had no noticeable inhibitory effect on erythrocyte invasion, although. they reacted with merozoite extracts in indirect ELISA and recognized the parasite surface in IFAT (Figs. 2 and 3). Antibodies directed against full size rMSA-2c, on the other hand, significantly neutralize invasion, according to the results shown in Table 2 and previous reports (Wilkowsky et al., 2003; Mosqueda et al., 2002). Thus, it is possible to hypothesize that (i) the protein regions represented by Peptides 1 and 4 are not critical for invasion, (ii) MSA-2c changes its tridimensional structure before invasion in such a way that these B-cell epitopes become cryptic and are not reachable by antibodies, or (iii) there are conformational B-cell epitopes that participate in the invasion process and are formed in the rMSA-2c protein, as some possible explanations for this event.

It is important to note that the lack of neutralization activity of sera against Peptides 1 and 4 rules out the possibility that the observed effects with anti-peptide 2 and 3 sera were due to cross-reactivity between KLH and *B. bovis* epitopes.

The algorithm used probably has a somewhat reduced sensitivity, since it did not predict as a B-cell epitope the region of the Mo7 MSA-2a1 mapped as the recognition site of the previously characterized mAb 23/70.174 (Goff et al., 1988; Reduker et al., 1989; Palmer et al., 1991; Jasmer et al., 1992; Hines et al., 1992). In any case, the methods employed in our work proved useful to identify B-cell epitopes in *B. bovis* MSA-2 proteins, two of which turned out to be sensitive to neutralization.

The line of research started with this work can lead to the production of chimaeric recombinant proteins composed of aligned neutralization-sensitive and surface exposed B- and relevant T-cell epitopes. These proteins could serve as design vaccines that might generate protective immunity against *B. bovis* in cattle, in a similar way as has been observed in the case of *P. falciparum* in malarial animal models (Shi et al., 1999, 2000; Collins et al., 2005; Zhou et al., 2006; Ravi et al., 2008.).

Conflict of interest statement

None declared.

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